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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Gary Ruvkun et al.	Art Unit:	1632
Serial No.:	08/908,453	Examiner:	R. Shukla
Filed:	August 7, 1997	Customer No.:	21559
Title:	AGE-1 POLYPEPTIDES AND RELATED MOLECULES AND METHODS		

SUBMISSION OF EXECUTED DECLARATION FROM DR. GARY RUVKUN

Applicants submit herewith an executed Declaration from Dr. Gary Ruvkun.

If there are any charges, or any credits, please apply them to Deposit Account No. 03-2095.

Respectfully submitted,

Date: 12 March 2002

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Gary Ruvkun et al.

Serial No.: 08/908,453

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Title: AGE-1 POLYPEPTIDES AND RELATED MOLECULES AND METHODS

Art Unit: 1632

Examiner: R. Shukla

Customer No.: 21559

Assistant Commissioner For Patents
Washington, DC 20231DECLARATION OF DR. GARY RUVKUN UNDER 37 C.F.R. § 1.132
TRAVERSING GROUNDS OF REJECTION

Under 37 C.F.R. §1.132, I declare:

1. I am an inventor of the subject matter described and claimed in the above-captioned patent application.
2. I have read the Office Action mailed on June 7, 2001.
3. The conclusion that *age-1* is a PI 3-kinase is supported by at least the following evidence: (i) sequence homology and characteristic structural motifs, (ii) physical interactions, (iii) genetic interactions, and (iv) biochemical evidence.
4. *age-1* shares sequence homology with other PI 3-kinases. *C. elegans* AGE-1 is the worm protein most homologous to known PI 3- kinases from mammals, and AGE-1

displays structural motifs characteristic of PI 3-kinases, including a p85 interaction domain and a lipid kinase domain. The closest mammalian homolog of *C. elegans* AGE-1 is mammalian p110 PI 3-kinase. When the p110 sequence was used to search the worm proteome database, AGE-1 was found to be p110's closest homolog. The random probability of alignment of AGE-1 with mammalian p110 kinase was extremely low, less than e^{-100} . Moreover, when the same search was conducted with a mammalian p110 PI 3-kinase query (for example, XP_066258, phosphoinositide 3-hydroxykinase p110-alpha subunit *Homo sapiens*), the next closest sequence hit in the *C. elegans* proteome database was 30-logs lower in probability, an enormous step down in sequence alignment terms. In addition, when the *C. elegans* AGE-1 sequence was used to search a mammalian proteome database, mammalian PI 3-kinases were also found to be AGE-1's closest homologs. Again, the random probability of this alignment to occur by chance rather than to reflect true orthology was extremely low, less than e^{-98} . These results provide strong evidence that *age-1* is the *C. elegans* ortholog of biochemically characterized mammalian PI 3-kinases.

Also consistent with these high levels of sequence similarity, we have found that substitution of an amino acid conserved between the AGE-1 polypeptide and mammalian p110 PI 3-kinase leads to complete loss of AGE-1 activity. This result lends further credence to the biological relevance of the sequence shared between AGE-1 and mammalian p110.

5. As a PI 3-kinase, AGE-1 would be expected to exhibit the characteristic physical interactions of a PI 3-kinase. PI 3-kinases are heterodimeric enzymes that

consist of a catalytic subunit, p110, and an SH2-domain-containing adapter subunit. In vertebrates p110 interacts with p85 or p55, SH2-domain-containing adapter subunits, through its amino-terminal domain; this interaction is required to activate the catalytic activity of p110. Examining the AGE-1 sequence, the amino-terminal domain of AGE-1 and p110 are 25% identical, suggesting that this interaction domain is under selective pressure to remain the same, perhaps due to the presence of a common regulatory partner, such as p85 or p55.

We used a BLAST search of the *C. elegans* genome to identify p85 or p55 homologs. This search identified a single *C. elegans* polynucleotide, *y110a7a-2.k*, that shared significant sequence homology (random probability of alignment: 3.8×10^{-29}) with mammalian p85 and p55. *y110a7a-2.k* encodes a p55-like adapter subunit, and was subsequently renamed *aap-1* for *age-1* adapter protein-1. Sequence comparisons using BLAST and PILEUP algorithms (Genetics Computer Group, WI) showed that the structure of AAP-1 was most closely related to SH2 domains from other Class IA PI 3-kinase adapter subunits. Based on the mammalian PI 3-kinase interactions, we predicted that the AAP-1 PI 3-kinase adapter subunit would bind to the amino-terminal domain of AGE-1.

We tested this prediction by producing recombinant AAP-1 and assaying for AAP-1 binding to the amino-terminal domain of AGE-1. AGE-1 (amino acids 1-268) was efficiently co-precipitated by AAP-1-containing beads; AGE-1 failed to bind to beads lacking AAP-1. These results indicated that AGE-1 specifically interacts with AAP-1, and confirmed our prediction that the interaction between mammalian p110 and

p85 is conserved in their *C. elegans* counterparts, AGE-1 and AAP-1. AGE-1's interaction with AAP-1 also provides support for AGE-1's identification as a PI 3-kinase, because of its shared physical interaction with SH2 adapter proteins.

This physical interaction was further demonstrated by the following *in vivo* experiment. If *aap-1* encodes the authentic regulatory subunit for AGE-1 PI 3-kinase, then *aap-1* gene function should be required for *age-1* function *in vivo*. To test this prediction, RNA-mediated interference was used to reduce *aap-1* gene function. Specifically, in *C. elegans*, injection of double-stranded RNA of a target gene results in RNA-mediated interference (RNAi) with target gene expression. This interference effectively reduces or eliminates the gene's activity in the injected animal and its progeny.

In our experiment, RNAi was used to test the prediction that animals with decreased *aap-1* function would resemble *age-1* loss-of-function mutants, *i.e.*, display constitutive arrest at the dauer larval stage. These studies were carried out in a sensitized genetic background that allowed the detection of small decrements in *aap-1* activity. We found that, in a sensitized background, *aap-1* RNAi strongly enhanced dauer arrest (compared to that observed for uninjected control animals), as expected if *aap-1* gene function is required for *age-1* function *in vivo*.

This physical and genetic evidence indicates that the SH2-domain-containing adapter protein, AAP-1, interacts with AGE-1, consistent with the interaction of their mammalian homologs and consistent with the identification of AGE-1 as a PI 3-kinase.

6. Additional evidence that AGE-1 is a PI 3-kinase is provided by genetic interactions that place AGE-1 in the insulin pathway. Vertebrate PI 3-kinases function in insulin signaling. If AGE-1 functions as a PI 3-kinase, then by analogy to mammalian systems AGE-1 would be predicted to act downstream of the *C. elegans* insulin receptor tyrosine kinase, which has specific phosphotyrosine motifs (YXXM) associated with p85 SH2-domain binding. We tested this prediction genetically and found that, not only does AGE-1 function downstream of the *C. elegans* insulin receptor, but AGE-1 also functions upstream of PDK and AKT kinases. This is relevant because PDK and AKT kinases have pleckstrin homology domains that are specifically regulated by the product of AGE-1, that is, PIP3.

Moreover, our genetic studies with *daf-18*, another component of the insulin-like signaling pathway, provide further support for AGE-1's identification as a PI 3-kinase. *daf-18*, the *C. elegans* homolog of mammalian PTEN, encodes a lipid phosphatase that dephosphorylates phosphoinositides *in vitro* and lowers PIP3 levels *in vivo* by inhibiting PIP3 accumulation in response to insulin signaling. Since PTEN dephosphorylates PIP3, DAF-18 may normally function to decrease the PIP3 output of AGE-1 PI 3-kinase signaling. If so, mutations in *daf-18* should suppress mutations in *age-1*. In our experiments, this hypothesis was found to be correct; we determined that mutations in *daf-18* suppressed mutations in *age-1*. Moreover, we found that loss of DAF-18 enhanced PIP3 signaling to

AKT kinases, consistent with AGE-1 generating a PIP3 second messenger and again consistent with AGE-1's role as a PI 3-kinase.

7. Finally, as biochemical evidence that AGE-1 is a PI 3-kinase, we again direct the Examiner's attention to the previously submitted publication by Babar et al. (*Neurobiology of Aging* 20:513, 1999). In this reference, the authors treated *C. elegans* with a known chemical inhibitor of mammalian PI 3-kinases, a chemical termed LY294002. This treatment mimicked the effects of AGE-1 mutations (pages 516-517), as measured by dauer formation, thermotolerance, and life span. This experiment indicates that the *in vivo* outcome of a loss of AGE-1 function parallels the *in vivo* outcome of a loss of PI 3-kinase activity. This biochemical result is therefore consistent with AGE-1 functioning as a PI 3-kinase.

8. It is reasonable to believe that PI 3-kinase activity may be readily assayed in *C. elegans* extracts. To prepare a *C. elegans* homogenate for a PI 3-kinase assay, standard methods of extract preparation are employed. In particular, worms are washed and concentrated, followed by homogenization to break open their outer cuticles. The homogenate is then centrifuged to remove debris, and the extract is used in a standard PI 3-kinase assay, such as the assay referenced in the specification at page 35, lines 25 and 26. Alternatively, if desired, AGE-1 protein may be purified from the crude homogenate using routine methods of protein purification. All of these methods were standard in the art at the time the patent application was filed.

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9. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

Date:

3/8/02
Dr. Gary Ruvkun

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TIBS 22 - JULY 1997

Phosphoinositide 3-kinases: a conserved family of signal transducers

Bart Vanhaesebroeck, Sally J. Leever,
George Panayotou and Michael D. Waterfield

Phosphoinositide 3-kinases (PI3Ks) generate lipids that are implicated in receptor-stimulated signalling and in the regulation of membrane traffic. Several distinct classes of PI3Ks have now been identified that have been conserved throughout eukaryotic evolution. Potential signalling pathways downstream of PI3Ks have been elucidated and PI3K function is now being characterised in several model organisms.

MEMBRANE LIPIDS DO not only have a structural role, but are also involved in signalling processes. A well-known example of this is the hydrolysis of phosphatidylinositol (4,5)-bisphosphate [$\text{PtdIns}(4,5)\text{P}_2$] by phospholipase C (Fig. 1), giving rise to diacylglycerol and inositol(1,4,5) P_3 , and to subsequent intracellular Ca^{2+} release and protein kinase C (PKC) activation¹. The lipids in the $\text{PtdIns}(4,5)\text{P}_2$ pathway are also substrates for phosphoinositide 3-kinases (PI3Ks), which phosphorylate the hydroxyl group at position 3 on the inositol ring and induce different signals (Fig. 1). This review will focus on three areas of PI3K research in which substantial progress has recently been made. First, we will describe the structural features and classification of the different PI3Ks. Next, we will review recently identified molecules that act upstream and downstream of PI3Ks. Finally, we will describe work on PI3K in different eukaryotic organisms, and discuss how these studies may contribute to our future understanding of the function of PI3K signalling.

PI3Ks generate three different lipids

PI3Ks convert PtdIns , $\text{PtdIns}(4)\text{P}$ and $\text{PtdIns}(4,5)\text{P}_2$ to $\text{PtdIns}(3)\text{P}$, $\text{PtdIns}(3,4)\text{P}_2$ and $\text{PtdIns}(3,4,5)\text{P}_3$, respectively (Fig. 1).

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$\text{PtdIns}(3)\text{P}$ is constitutively present in eukaryotic cells and its levels are largely unaltered upon cellular stimulation. By contrast, $\text{PtdIns}(3,4)\text{P}_2$ and $\text{PtdIns}(3,4,5)\text{P}_3$ are almost absent from resting cells. Their intracellular concentration rises sharply upon stimulation with a variety of ligands, suggesting a likely function as second messengers (reviewed in Ref. 2). PI3K lipid products are not substrates for phospholipases, so they are not degraded into soluble inositol phosphates. Instead, phosphatases mediate their catabolism by removing the phosphate group at position 3 or 5 of the inositol ring^{2,3} (Fig. 1). Several 5-phosphatase genes have now been cloned (for overview, see Ref. 3) and their role in signalling is being elucidated (for example, see Ref. 4).

PI3Ks fall into three classes

PI3K catalytic subunits can be divided into three main classes on the basis of their *in vitro* lipid substrate specificity, structure and likely mode of regulation (Table 1; see also Ref. 5).

Class I PI3Ks phosphorylate PtdIns , $\text{PtdIns}(4)\text{P}$ and $\text{PtdIns}(4,5)\text{P}_2$. *In vivo*, however, their preferred substrate is likely to be $\text{PtdIns}(4,5)\text{P}_2$. All mammalian PI3Ks from this class interact with active, GTP-bound Ras (Refs 6–10; R. Wetzker, pers. commun.). They all form heterodimeric complexes with adaptor proteins that link them to different upstream signalling events. Class I PI3K catalytic subunits can be subdivided into two subclasses (A and B) according to the type of adaptor subunit with which they associate.

Class I_A PI3Ks are 110–130 kDa proteins that interact with adaptor α subunits containing src homology-2 (SH2) domains. These adaptors bind phosphorylated Tyr residues, thereby linking class I_A PI3K catalytic subunits to Tyr kinase signalling pathways. Class I_A PI3K catalytic subunits include mammalian p110 α , β and δ , and homologous molecules from several other species (Table 1). They contain several conserved regions including the adaptor- and the Ras-binding sites, the PI-kinase region (PIK; of unknown function, also found in PI4Ks) and the carboxy-terminal kinase domain⁵.

To date, eight different adaptor subunits for class I_A catalytic subunits have been described (seven in mammals encoded by three different genes, and one in *Drosophila*; see Fig. 2). They all contain two SH2 domains linked by an inter-SH2 region, which is both necessary and sufficient for binding to the catalytic subunits. The SH2 domains bind phosphorylated Tyr residues, specifically within a pTyr-x-x-Met motif. The 85 kDa adaptors also contain an SH3 domain and a breakpoint cluster region (BCR)-homology domain (BH), whose precise binding partners and/or regulatory role are unclear. There has been no report to date of a preferential coupling between any of the class I_A adaptors and catalytic subunits, although it is possible that tissue-specific differences in function or regulation may exist.

Class I_B PI3Ks are stimulated by G-protein $\beta\gamma$ subunits, and do not interact with the SH2-domain-containing adaptors that bind to class I_A PI3Ks (Table 1). The first identified member of this PI3K subfamily, p110 γ (Ref. 11) contains an amino-terminal Ras-binding site, a PIK domain and a catalytic domain (Table 1). Stephens and co-workers recently reported the isolation of a regulatory p101 subunit that associates tightly with p110 γ (Ref. 12). This novel adaptor does not display any homology with known proteins and the exact mechanism by which it mediates coupling of p110 γ to G proteins remains unknown.

Class II PI3Ks are larger (> 200 kDa) enzymes that phosphorylate *in vitro* PtdIns and $\text{PtdIns}(4)\text{P}$, but not $\text{PtdIns}(4,5)\text{P}_2$. Their defining feature is a C2 domain¹³ at their carboxyl terminus (Table 1). The C2 domains of class II PI3Ks lack critical Asp residues that coordinate

*Nomenclature used is according to Ref. 1. Phosphoinositide (PI) is used as a generic term, whereas phosphatidylinositol (PtdIns) is used to indicate specific phosphoinositides, e.g. $\text{PtdIns}(3)\text{P}$.

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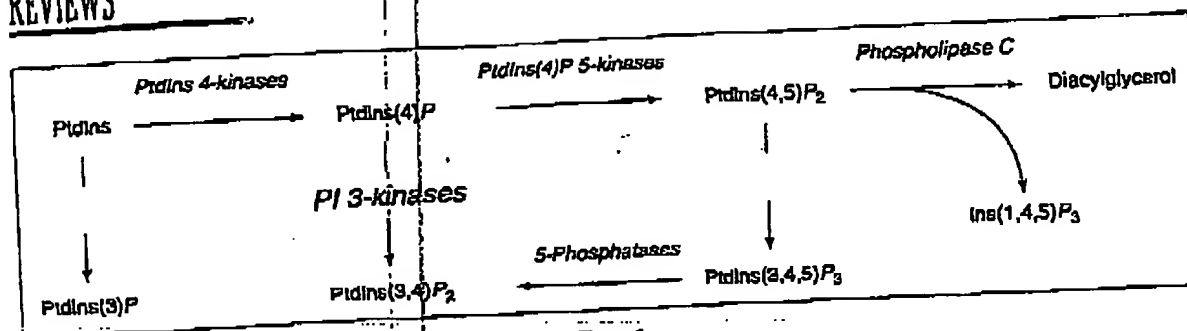


Figure 1

Phosphoinositide pathways. Ins, inositol; PI 3-kinases, phosphoinositide 3-kinases; PtdIns, phosphatidylinositol.

binding of Ca^{2+} in the first C2 domain of synaptotagmin¹³. Consistent with this, a class II PI3K has been found to bind lipids in a Ca^{2+} -independent manner¹⁴. At present, it is unknown whether class II PI3K activity is regulated by extracellular stimuli.

Class III PI3Ks have a substrate specificity restricted to PtdIns. These PI3Ks are homologous to Vps34p, the only PI3K present in yeast. Vps34p is essential for the trafficking of newly formed proteins from the Golgi to the vacuole, the equivalent of the mammalian lysosome (reviewed in Refs 3, 15, 16). Members of this class of PI3Ks also

occur as heterodimers. Yeast Vps34p is found in complex with Vps15p, a 170 kDa Ser/Thr kinase, which both activates and recruits Vps34p to membranes¹⁵. Similarly, human Vps34p associates with a 150 kDa Vps15p homologue¹⁷. The current hypothesis is that class III PI3Ks and their PtdIns(3)P lipid product fulfil a housekeeping role in constitutive membrane trafficking and vesicle morphogenesis^{3,15,16}. It is worth mentioning here that class I and II enzymes and their lipid products are also likely to have a function in vesicular trafficking, for example in post-endocytic sorting of ligand-stimulated receptors^{1,15,16}.

Signalling via PI3Ks

Recent advances in the understanding of signals feeding into and relayed by mammalian class I PI3Ks will be reviewed, focusing on the signalling molecules *per se*, without elaborating on the multiple biological responses (such as cytoskeletal rearrangements, cellular migration, mitogenesis, differentiation and protection from apoptosis) in which PI3Ks have been implicated (reviewed in Refs 18–20).

What happens upstream of class I PI3Ks? Class I PI3Ks are involved in signalling by the majority of receptors with intrinsic or associated (e.g. Src-like) Tyr kinase

Table 1. A classification of phosphoinositide 3-kinase (PI3K) family members

Class	In vitro lipid substrates and structural features of catalytic subunits ^a	Subunits ^b		Regulation
		Catalytic	'Adaptor'	
I	PtdIns, PtdIns(4)P, PtdIns(4,5)P ₂	p110 α , β , δ (m) Op110 (Dm) AGE-1 (Ce) PIK1, PIK2 (Dd)	p85 α , β (m) p55 α , γ (m) p50 α (m) p60 (Dm)	Tyr kinases and Ras
		p110 γ (m) PIK3 (Dd)	p101 (m)	G protein ($\beta\gamma$ subunits) and Ras
II	PtdIns, PtdIns(4)P	PI3K-C2 α /Cpk-m/p170 (m) PI3K-C2 β (m) PI3K-GBD/Cpk (Dm) PI3K-C2 (Ce)	?	?
III	PtdIns	Vps34p ^c	Vps15p (Sc) p150 (m)	Constitutive?

^aKey of structural motifs: adaptor-binding (light purple); Ras-binding (green); C2 (yellow); PIK (dark purple); Kinase domain (red).

^bFor the proteins other than those derived from yeast, fruit fly and mammals, no biochemical proof of PI3K lipid kinase activity is available. These enzymes have been allocated to a particular class of PI3K mainly based on primary sequence homology of the core kinase domain^b. The abbreviations used are: m, mammalian; Ce, *Caenorhabditis elegans*; Dd, *Drosophila melanogaster*; Dm, *Drosophila melanogaster*; Sc, *Saccharomyces cerevisiae*. The GenBank/EMBL accession numbers for class I and II catalytic subunits are: mammalian: p110 α (human: Y10743), p110 β (human: Y10055, U57843, U86587; mouse: U86453), PI3K-C2 α (human: Y13367), Cpk-m (human: S67334), p110 γ (human: X83988; pig: Y10743), p110 δ (human: Y10055, U57843, U86587; mouse: U86453), PI3K-C2 β (human: Y13367), Cpk-m (also known as p170) (mouse: U52193; U57772), PI3K-C2 β (Y13892, Y11312) – *D. melanogaster*: Op110 (Y09070), PI3K-GBD (also known as Cpk) (X32892; U23478), Accession numbers for p101, Vps15p and p150 are Y10742, M59835 and Y08991, respectively.

^cThe prototype of the class III PI3Ks is the *S. cerevisiae* protein Vps34p (X59331). Vps34p homologues from other species are not shown individually. They are: human PI3K (Z46973); *D. melanogaster*, PI3K-59R (X99912); *D. discolorum*, PIK5 (U23480); and the Vps34p-related PI3Ks from *Schizosaccharomyces pombe* (U32583), *Soybean* (U29770), *Arabidopsis thaliana* (U10689) and *C. elegans* (Y12543).

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activity, and by receptors linked to heterotrimeric G proteins (for a listing of the signals that trigger class I PI3K activation, see Refs 2, 21).

At present, it is unclear how G-protein signals are relayed from the plasma membrane to the class I₂ enzymes. For class I₁ PI3Ks, the phosphorylated Tyr residues, generated on receptors or associated substrate molecules (such as IRS-1/2 in signalling by insulin and cytokines) form the docking sites for the SH2 domains of the PI3K adaptor subunits. This adaptor-mediated translocation of PI3Ks to receptor Tyr kinases and their substrates is likely to help position the catalytic subunits close to membranes that contain their lipid substrates.

In addition, class I PI3Ks interact with Ras proteins in a GTP-dependent manner. Thus far, this interaction has only been studied in detail for the p110 α -p85 α complex²¹⁻²³. The Ras-related proteins, Rac (which has been implicated in signalling downstream of PI3Ks²²) and Rho, do not bind p110 α -p85 α (Ref. 6). *In vitro*, incubation of GTP-Ras with p110 α -p85 α results in a modest increase in PI3K kinase activity⁴. Co-expression studies of p110 α -p85 α with various Ras mutants indicate that Ras can regulate p110 α -p85 α *in vivo*^{21,22} (Fig. 3). Evidence from experiments using platelet-derived growth factor (PDGF)-receptor mutants also suggests that accumulation of GTP-bound Ras is required for full activation of class I PI3Ks by PDGF²³. At present, however, it is not yet known to what extent PI3K activation by receptor Tyr kinases also occurs independently of Ras (Fig. 3).

Taken together, these data indicate that PI3Ks might be another class of Ras effector molecules, alongside proteins such as the Raf Ser/Thr kinases (reviewed in Ref. 24). The interaction of Ras with a PI3K might result in allosteric activation and/or contribute to PI3K recruitment to the plasma membrane. Interestingly, Ras effector mutants have been identified that interact with p110 α , but not with Raf-1 (and *vice versa*)²⁵ consistent with the existence of a Raf-independent signalling pathway downstream of Ras (see also below). It should be noted that data have also been reported that position Ras downstream of PI3Ks²⁶.

What happens downstream of PI3Ks? It is generally hypothesised that PI3K lipid products interact with certain proteins and modulate their localisation and/or activity. The recent characterisation of protein modules, such as pleckstrin-homology (PH)²⁷ and C2 domains, which can bind lipids, supports this view. If se-

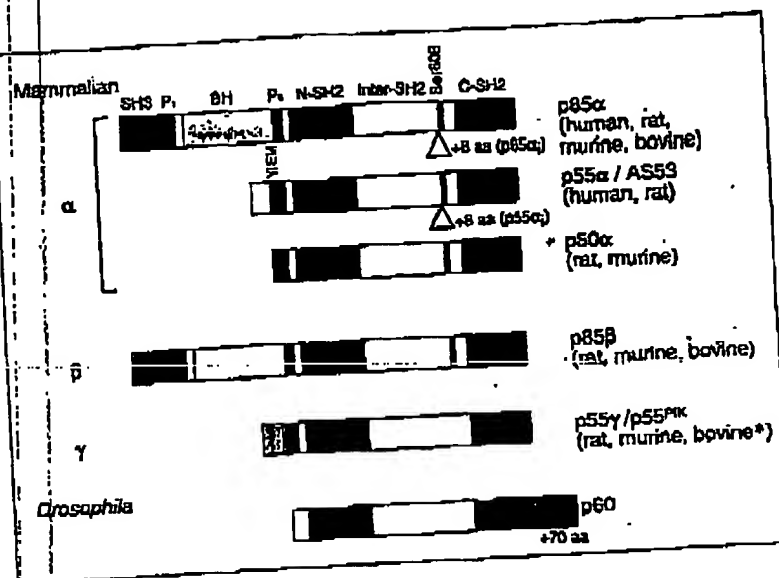


Figure 2

Figure 2
Overview of the different adaptor subunits for class I_h phosphoinositide 3-kinases. P, Pro-rich region; BH, bcr homology region. p50 α and p55 α (also known as p85/AS53) are splice variants of p85 α , whereas p85 β and p55 γ (also indicated as p55TM) are encoded by different genes. Triangles indicate further splice insertions in p85 α and p55 α (here named p85 α and p55 α). Possible regulatory phosphorylation sites are indicated as Ser608 (p85 α and p55 α). *Bovine p55 γ (M. D. Waterfield et al., unpublished).

lective targets downstream of receptor-stimulated PI3Ks exist, they are expected to have very high affinity and specificity for $\text{PtdIns}(3,4)\text{P}_2$ and/or $\text{PtdIns}(3,4,5)\text{P}_3$ over $\text{PtdIns}(4,5)\text{P}_2$ because the latter lipid is estimated to be at least 10–100-times more abundant in most stimulated cells¹⁶. The observation that certain FH domains seem to bind specifically to either $\text{PtdIns}(4,5)\text{P}_2$ or $\text{PtdIns}(3,4,5)\text{P}_3$ is consistent with such a hypothesis²⁵. However, another important consideration is that several PI3Ks possess intrinsic protein kinase activity, which might be involved in PI3K signalling. Thus far, only auto- and intersubunit phosphorylation of PI3Ks has been documented and no *in vivo* protein kinase substrates for PI3Ks have been identified^{26,27}.

One postulated function for PI3Ks is in cytoskeletal reorganisation via exchange factors that regulate the small GTP-binding protein Rac²², and that modulate the affinity of integrins for the extracellular matrix²². In addition, several protein Ser/Thr kinases have been placed downstream of PI3Ks in receptor-stimulated signalling, including Akt [also termed protein kinase B (PKB) or RAC-PK (Related to PKA and PKC protein kinases)], p70 ribosomal S6 kinase (p70^{S6K}) and PKC (Fig. 3).

(1) Akt and its target GSK3. The Akt Ser/Thr protein kinases, the cellular homologues of the retroviral oncogene *v-akt*, are activated upon receptor-Tyr kinase stimulation (reviewed in Ref. 31). The three mammalian Akt molecules identified to date are all composed of an amino-terminal PH domain, followed by a catalytic domain and a small (~70 amino acids) carboxy-terminal extension, which lacks sequence homology to other proteins.

The activation of Akt most likely involves specific phosphorylations as well as a PH-domain mediated lipid binding. However, while it is clear that Akt can bind lipids, the specificity of this event and its effect on the activity and on the intracellular localisation of Akt remain controversial^{31,32}. Upon stimulation of cells with insulin, two sites in Akt-1 (Thr308 in the catalytic domain and Ser473 in the carboxy-terminal tail) become phosphorylated *in vivo* in a PI3K-sensitive manner³³ (Fig. 3). Phosphorylation of both residues appears to be critical for high-level activity of Akt, suggesting that one or more upstream kinases activate Akt *in vivo*. Recently, a protein kinase activity has been purified which phosphorylates Akt at Thr308

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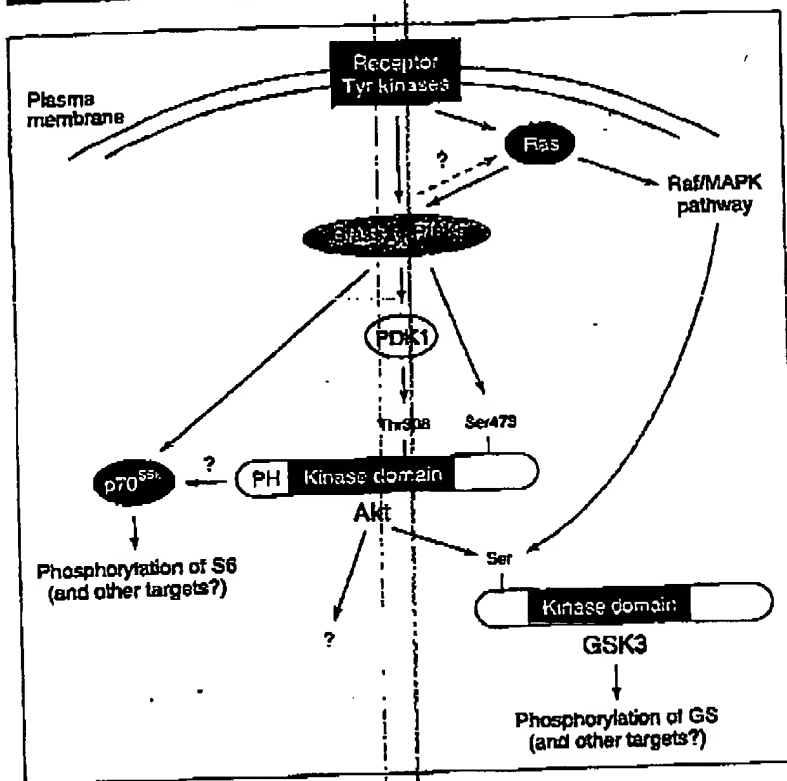


Figure 3

Signalling pathways linked to mammalian class I phosphoinositide 3-kinases. Arrows only denote a signalling pathway without specifying whether intermediary signalling molecules are involved. Abbreviations used: GSK3, glycogen synthase kinase-3; MAPK, mitogen-activated protein kinase; p70^{S6}, p70 S6/kinase; PDK, PtdIns(3,4)P₂/PtdIns(3,4,5)P₃-dependent kinase; PH, pleckstrin-homology domain.

(but not Ser473) *in vivo*³². Remarkably, the activity of this kinase is uniquely dependent on the presence of PtdIns(3,4,5)P₃ or PtdIns(3,4)P₂, and has therefore been named PtdIns(3,4)P₂/(3,4,5)P₃-dependent kinase-1 (PDK1).

Given that Akt is one of the most likely downstream targets of PI3Ks identified so far, and that Akt seems to play a central role in the PI3K-mediated protection against apoptosis²⁰, it is important to know what lies further downstream on this pathway (Fig. 3). There is some evidence to position p70^{S6} (see below) downstream of Akt²⁴. To date, however, the only known substrate of Akt *in vivo* is glycogen synthase kinase-3 (GSK3). Upon stimulation of cells with insulin, Akt phosphorylates GSK3 on a single, conserved regulatory amino-terminal Ser in a PI3K-sensitive manner²⁵. Phosphorylation and consequent inactivation of GSK3 results in the dephosphorylation and activation of a spectrum of metabolic and gene-regulatory proteins (reviewed in Ref. 36). Therefore, this link may turn out to be crucial for PI3K to

exert its varied downstream effects. It should be mentioned that, in addition to PI3K and Akt, the Raf/mitogen-activated protein kinase (MAPK) pathway has also been implicated in GSK3 regulation by growth factors other than insulin (such as epidermal growth factor, reviewed in Ref. 36).

(2) p70^{S6} becomes activated upon mitogenic stimuli and plays an important role in the progression of cells from G1 to S phase of the cell cycle. It phosphorylates the S6 protein component of the 40S ribosomal subunit during mitogenic responses, but might also be involved in the regulation of other cellular processes (reviewed in Refs 37–39). The role of S6 phosphorylation is still not fully understood, but correlates with an increase in translation, probably from specific mRNAs encoding proteins essential for G1 progression (reviewed in Ref. 39).

Activation of p70^{S6} is regulated by multiple independent Ser/Thr-directed phosphorylations. This activation is independent of the Raf/MAPK pathway, but involves PI3Ks and the PIK-related

kinase, mTOR (for mammalian-target of rapamycin), as well as PKC and as yet unidentified proline-directed kinases (reviewed in Refs 37–39). However, kinases that directly phosphorylate and activate p70^{S6} *in vivo* have not been identified.

(3) PKC/PRK. Both the lipid substrates and products of PI3Ks have been reported to activate, *in vitro*, a broad panel of PKC family members and PKC-related kinases [PRK, also indicated as protein kinases N (PKN)]⁴⁰. Although one group has shown that both PtdIns(4,5)P₂ and PtdIns(3,4,5)P₃ activate these kinases to the same extent, other groups (cited in Ref. 40) have reported conflicting data, which may be attributed to differences in lipid presentation procedures. Whereas PKCs may indeed be affected by PI3K lipid products, there is no consensus as to which member of this family, if any, is a selective target of these lipids.

Model systems in different organisms

In addition to mammals, PI3K genes have been identified in other organisms including yeasts, plants, slime molds, nematodes and fruit flies. Although the analysis of PI3K function in most of these organisms is at an early stage, future work applying genetic techniques should complement the mammalian studies in several respects. Most importantly, a genetic approach should allow the identification and characterisation of interacting genes, and improve our understanding of the function of different PI3Ks at the level of a cell, organ or entire organism. Below, we summarise the genetic systems currently emerging that are likely to play an important role in future studies of PI3K function.

Yeasts possess only class III PI3Ks and have already provided a useful model system to examine the function of this PI3K class. As described above, studies in *S. cerevisiae* and in mammalian cells have been complementary and suggest a ubiquitous role in constitutive vesicular trafficking¹⁵.

The slime mold *A. discoidium* is a motile chemotactic unicellular organism that can form multicellular aggregates and fruiting bodies under conditions of nutritional stress. Four putative *A. discoidium* PI3K genes have been identified⁴¹. DdPIK1 and -2 resemble class I₁ PI3Ks; DdPIK3 is most closely related to the class I₂ group of G-protein-activated PI3Ks; and DdPIK5 closely resembles class III Yps34p-like PI3Ks. Separate disruption of each class I PI3K gene has no detectable phenotype whereas simultaneous disruption

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of both class I_A PI3Ks has pleiotropic effects on growth and development, suggesting a certain level of redundancy⁴¹. By contrast, removal of the class III PI3K is lethal, as is removal of the class I_B PI3K together with either of the class I_A PI3Ks.

The nematode *C. elegans* is a more complex multicellular eukaryote with specialised terminally differentiated cells. *C. elegans* possesses genes encoding one PI3K from each class, though only the function of the class I_A PI3K homologue has been studied to date⁴². The class I_A PI3K gene, termed *age-1* or *daf-23*, was identified in genetic screens for mutants that promote longevity (*age* mutants)⁴³ and for mutants that affect dauer larvae formation (*daf* mutants)⁴⁴. Under uncrowded conditions with ample food, wild-type *C. elegans* develop rapidly through four larval stages (L1-L4) to become adult worms with a life span of 2-3 weeks (Fig. 4). By contrast, when food is scarce and the population density high, an alternative third stage larva, the dauer larva, is formed. Dauer larvae are developmentally arrested, non-feeding and can endure harsh environmental conditions. When more favourable conditions return, dauers recover and develop into adults with a normal life span, irrespective of how much time has been spent as a dauer.

Null mutations in the *age-1* PI3K result in *C. elegans* that form dauer larvae constitutively, suggesting that *age-1* normally suppresses dauer formation under favourable conditions. Alternatively, *age-1* may be required for L3 development such that in the absence of *age-1* activity, dauer larva development occurs by default. Presumably, when nutrients are depleted, *age-1* is inactivated and an altered developmental programme that leads to dauer formation is initiated. *C. elegans* with reduced levels of the *age-1* PI3K (e.g. with maternal, but not zygotic *age-1* PI3K, or carrying weak mutant alleles of *age-1*) do not form dauer larvae, but develop into adults with significantly extended adult lifespans^{45,46} (Fig. 4). A plausible explanation for this link between the *daf* and *age* phenotypes is that the genes normally expressed in the dauer larvae to make them long-lived and resistant to environmental stress are inappropriately expressed in adults with reduced levels of *age-1*, thereby conferring increased longevity and stress resistance^{44,46}.

The analysis of genetic interactions between *age-1* and various *daf* mutants has facilitated their ordering into a complex

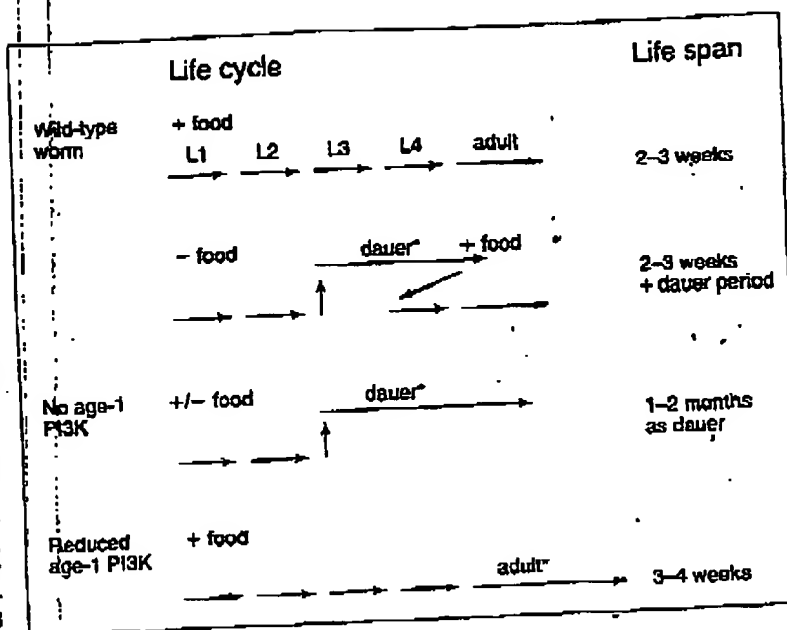


Figure 4

Mutations in *age-1* phosphoinositide 3-kinase affect the life cycle of *Caenorhabditis elegans*. A scheme depicting the effects of loss of function mutations in the *age-1* PI3K gene on the nematode life cycle. The four larval stages (L1-L4) and the dauer larva, are shown. + food indicates that worms were growing in uncrowded conditions with ample food; - food indicates that worms were growing in over-crowded conditions with limited food; * indicates life cycle stages with increased resistance to environmental stress.

pathway affecting dauer formation and life span^{47,48,49}. Characterisation of these genes at the molecular level should help to clarify the way in which the *age-1* PI3K functions as a signalling molecule. Mutations in *daf-2* also result in both constitutive dauer formation and increased adult longevity, while mutations in *daf-16* suppress both the dauer constitutive and increased longevity phenotypes resulting from mutations in *age-1* and *daf-2*. It will be intriguing to discover what the *daf-2* and *daf-16* genes encode.

The fruit fly *D. melanogaster* has genes encoding one PI3K from each class¹⁴, so should also serve as a useful model system with which to address the function of the different PI3Ks. The *D. melanogaster* class I_A PI3K, Dp110 (Ref. 49; Table I) associates with p60, an SH2 domain-containing adaptor (Fig. 2), which, like its mammalian counterparts, recognises the pTyr-x-x-Met motif⁵⁰. Ectopic expression studies have suggested that Dp110 might play a role in the control of cell growth⁴⁹. The overproduction of Dp110 in wing or eye imaginal discs (sheaths of epithelial cells which expand and differentiate during larval growth and ultimately give rise to the structures that make up the adult fly) results in adult flies with enlarged wings

or eyes, whereas overproduction of a dominant-negative version of Dp110 inhibits the growth of these structures⁴⁹. Interestingly, loss of function mutations in the *D. melanogaster* homologue of the insulin receptor, *InsR*, also inhibits imaginal disc cell growth and result in the generation of smaller-than-wild-type flies⁵¹. So, in flies as well as mammals, class I_A PI3Ks might be important targets of the insulin receptor, and may regulate normal growth during development.

Concluding remarks

In recent years, a multitude of PI3Ks have been identified with unique molecular and biochemical characteristics indicating that they fall into distinct PI3K classes. The challenge now is to assign biological roles to each of these classes and to determine whether the lipids generated by the different classes of PI3Ks exert selective or redundant biological functions. One crucial issue is to find how 3-phosphorylated lipids mechanistically affect downstream signalling. To fully understand the biological events brought about by PI3K signalling, there is clearly a need to combine a variety of experimental approaches including pharmacology, biochemistry, cell biology and genetics.

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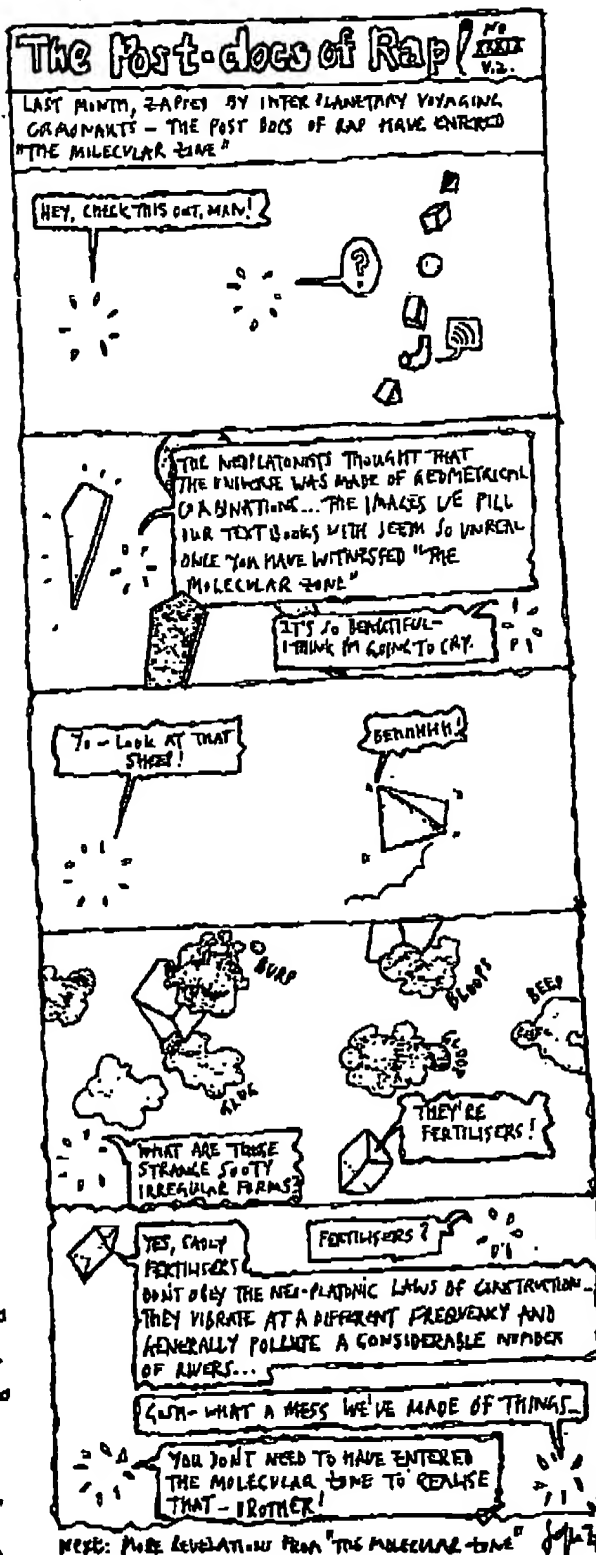
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Genetic pathways that regulate ageing in model organisms

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Searches for genes involved in the ageing process have been made in genetically tractable model organisms such as yeast, the nematode *Caenorhabditis elegans*, *Drosophila melanogaster* fruitflies and mice. These genetic studies have established that ageing is indeed regulated by specific genes, and have allowed an analysis of the pathways involved, linking physiology, signal transduction and gene regulation. Intriguing similarities in the phenotypes of many of these mutants indicate that the mutations may also perturb regulatory systems that control ageing in higher organisms.

The propagation of a species could depend in principle either on the perfect maintenance of its individuals or on the ability to renew the population with young members. Nature most commonly has chosen the latter option.

Renewal is typically achieved by the setting aside of a pristine lineage of genetic information — that is, the germ line — which is passed on by sexual reproduction. In contrast, the somatic lineage of all animals declines and degenerates with age, giving rise to phenotypic changes recognized as ageing. Studies in model organisms have begun to map out important genes and pathways that seem to regulate the pace of ageing and that are remarkably conserved from yeast to metazoans. These studies have the great advantage that one can use genetic approaches to search directly for mutations that change life span. This makes it possible to identify mechanisms in a way that is independent of any preconceived model of ageing. Recently, the analysis of such mutations that affect life span has revealed several different pathways that influence the ageing process. Our review will focus largely on insights into ageing that have been gleaned from analysing certain single-gene mutations in the two model organisms in which the molecular mechanisms of ageing are best understood, yeast and the nematode worm *C. elegans*. Studies reveal surprisingly simple and conserved mechanisms governing the pace of ageing and life span. Although it is too early to extrapolate these findings to mammals, it seems likely that they will spark molecular insights into causes of human ageing.

Caloric restriction

One of the best indications that the ageing process is subject to regulation is that life span can be extended by caloric restriction¹. Caloric restriction typically refers to a diet in which calories are limited by 30–40% compared with animals fed *ad libitum*. Caloric restriction extends life span in rodents, worms, yeast and probably primates. This response to caloric restriction has clear selective value, because it allows animals to postpone reproduction until food is available. However, when food is restored the animals can produce progeny, even when well-fed controls are post-reproductive or no longer alive. The mechanism by which caloric restriction extends life span is unclear. One hypothesis is that caloric restriction slows metabolism, thereby slowing the production of toxic reactive

oxygen species and, in turn, slowing ageing. Although this idea is consistent with a probable link between oxidative damage and ageing², it has never been validated experimentally. Other examples reinforce the notion that within a species there is a relationship between metabolic rate and ageing³. For example, *Drosophila* live much longer when maintained at lower temperatures. However, there is every reason to think that the correlation between metabolic rate and ageing can be broken by genetic changes. For example, bats have a comparable metabolic rate to mice, yet live more than ten times longer. In addition, single-gene mutations in yeast, *C. elegans*, *Drosophila* and mice can extend the life span significantly without an apparent slowing in metabolism. Thus it is reasonable to conclude that metabolic rate is important within an individual or species, but its impact may be overridden by evolutionary divergence (bats compared with mice) or even single-gene mutations conferring increased or greater longevity. It is interesting to speculate that mutations that break this correlation might alter the regulatory machinery that couples caloric restriction and ageing.

Gene silencing as a regulator of life span

Studies of ageing in the budding yeast *Saccharomyces cerevisiae* have led to the conclusion that a gene involved in the silencing of chromatin may be a key regulator of ageing. Silencing is a process by which entire regions of chromosomes encompassing blocks of genes are rendered transcriptionally inactive. In budding yeast the life span is measured by the number of cell divisions in a mother cell lineage^{4–6}, determined by microscopic manipulation (Fig. 1). Mother cells divide a relatively fixed number of times and undergo characteristic changes as they age. Although yeast seem to violate the rule of a demarcation of soma and germ line, closer inspection reveals a renewal process in the budding of daughter cells, from an ageing, soma-like lineage of mother cells. A phenotypic change that occurs in ageing mother cells is the expression of the repositories of a and α mating-type genes owing to a breakdown in genomic silencing at the *HML* and *HMR* loci where extra copies of this structural information is stored. Haploid cells normally express either a or α information from a mating-type locus termed *MAT*. The breakdown in silencing at *HML* and *HMR* leads to the simultaneous expression of genes of both mating types thereby resulting in sterility⁷. Other phenotypic changes evinced by mother cells as they

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age include cellular enlargement and a slowing of the cell cycle. Gross changes in the morphology of the nuclei also occur, most notably a massive expansion in the size of the nucleolus (described in more detail below). Finally, bud scars, which are the remnant of each cellular division, accumulate on the surface of ageing mother cells.

Silencing in yeast is carried out by a complex of silent information regulator (Sir) proteins — Sir2, Sir3 and Sir4 — at telomeres and mating-type genes¹⁰. A causal link between silencing and ageing was first made by the identification of the *SIR4-42* longevity mutation¹⁰, which redirects the Sir2/3/4 complex away from telomeres and *HM* loci to the region of the genome encoding the ribosomal RNA (rDNA). The rDNA contains 100–200 tandem repeats of genes encoding the large and small rRNAs and organizes the nucleolus as the site of ribosome synthesis. Sir2 normally mediates silencing in the rDNA without the other Sir proteins^{11,12}. Subsequent experiments validated the notion that the amount of Sir2 at the rDNA was predictive of life span; *sir2*-deletion strains have a short life span, and strains with an extra *SIR2* copy have super-extended life spans¹³. Thus an increase in rDNA silencing by Sir2 seems to increase life span. It has also been reported that deletion of the Rpd3 global histone deacetylase, which removes the acetyl groups on lysines of the amino termini of histones, extends life span¹⁴. Initially this finding seems strange, as the increase in acetylation of histones resulting from the loss of a deacetylase will normally result in an increase in gene expression. However, consistent with the extension in life span, the loss of Rpd3 actually leads to an increase in rDNA silencing¹⁵. The reason why removal of the Rpd3 deacetylase decreases expression is not clear, but may be an indirect effect of an increased expression of genes encoding the Sir silencing proteins.

Silenced chromatin in the repeated rDNA array represses recombination¹⁶, which would otherwise generate extrachromosomal rDNA circles (ERCs) that accumulate in subsequent divisions in mother cell lineages and have been reported to be a cause of ageing¹⁷. But the importance of ERCs in ageing has been a matter of some controversy. On the one hand, the generation of ERCs early by a site-specific recombinase shortens life span in mother cells¹⁷. Moreover, deletion of *FOB1*, encoding a protein that promotes a unidirectional block in rDNA replication, reduces or eliminates the formation of ERCs and extends life span¹⁸. These findings implicate ERCs in ageing in yeast. On the other hand, the short-lived *sgs1* mutant, defective in the Werner-like DNA helicase (see review in this issue by Martin and Oshima, pages 263–266), was recently reported not to have an elevated level of ERCs¹⁹. In addition, other DNA circles, such as the *TRP1* ARS1 plasmid, can shorten life span¹⁷, indicating that ERCs are not unique in their ability to accumulate in and kill mother cells. Finally, mother cells that experience a sojourn in stationary phase showed a shortened life span without any increase in ERCs, implicating at least one ERC-independent mechanism in ageing²⁰. The most accurate surmise at present is that ERCs are one of several inputs causing ageing in wild-type mother cells. The short life span in *sgs1* mutants, however, seems to be more complicated than originally surmised²¹, and may involve other mechanisms.

SNF1 is a candidate for a second input into ageing, acting through a metabolic pathway that determines carbon-source utilization and energy levels in cells. *SNF1* encodes a kinase that activates genes required for utilization of certain carbon sources other than glucose²². Surprisingly, the activity of *SNF1* was found to increase with the age of mother cells, even in the presence of abundant glucose²³. This change is accompanied by a metabolic shift to energy storage resulting in increases in cellular ATP and nicotinamide adenine dinucleotide (NAD). These changes are linked closely to the ageing process because null mutations in the *SNF1* co-repressor, *SIP2*, decrease life span, and mutations in the *SNF1* co-activator, *SNF4*, increase life span. It is possible that, when phosphorylated, one or more *Snf1* targets cause accelerated ageing. The identification of such targets might suggest how this metabolic pathway is integrated with the silencing pathway above.

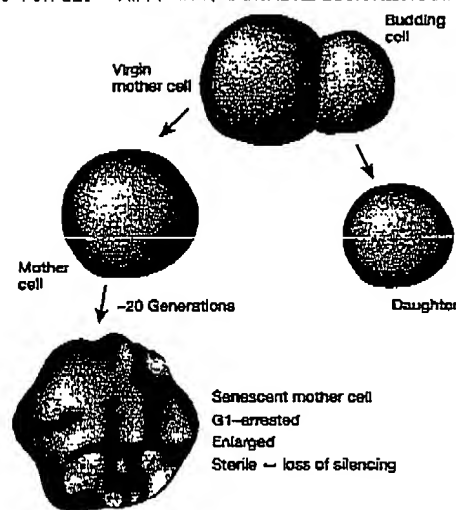


Figure 1 Ageing in budding yeast. Shown is the asymmetric budding of a virgin mother cell, that is, one that arose from a bud in the previous cell division. The cell division depicted gives rise to a mother cell and a daughter that arose from the bud. By following the mother-cell pedigree microscopically over many generations, changes in phenotype are observed, including cell enlargement and sterility (see text). This process leads to a loss of division potential after about 20 divisions yielding a senescent mother cell that has a bumpy, wrinkled appearance.

Sir2 proteins at the interface of ageing and metabolism

The importance of silencing in ageing raises the question of what is the exact biochemical function of Sir2. A core domain of Sir2 has been widely conserved from bacteria to humans^{24,25} and possesses a fascinating enzymatic activity. The early observation that overexpression of Sir2 caused the global deacetylation of histones led to the conjecture that this protein was a histone deacetylase²⁶. However, biochemical experiments showed that the purified protein displayed a different enzymatic activity, albeit a weak one, that of an ADP ribosyltransferase, using NAD as a donor^{23,27}. An apparent solution to this puzzle came with the demonstration that Sir2 does indeed require NAD, but this requirement is for the activation of a robust histone deacetylase activity²⁸. This activity displayed specificity for lysines 9 and 14 of histone H3 and lysine 16 of H4, residues shown to be important for silencing *in vivo*. This NAD-dependent deacetylase activity seems to be a universal property of Sir2 proteins from bacteria to mammals^{29–30}.

The NAD requirement for the Sir2 deacetylase is highly unusual and provocative in the context of ageing. NAD is more typically used in hundreds of metabolic reactions in cells to receive electrons or, in its reduced form NADH, to donate them. In the case of Sir2, NAD seems to function as a cofactor that activates the deacetylase, potentially coupling that activity to the energy status of cells. Sir2 proteins may in this way sense metabolic rate and transduce this energy status to life span by their deacetylase activities. The increase in NAD levels in normal ageing mentioned above²³ might represent a homeostatic response of cells to help oppose the waning of genomic silencing by the Sir proteins.

In this regard, caloric restriction can be modelled in yeast by limiting glucose concentrations from 2% to 0.5% in the growth media or by mutating components of the cyclic AMP-dependent protein kinase A (PKA) pathway, which signals glucose availability to cells (Fig. 2)³¹. Either regimen leads to a significant extension in the life span of mother cells. Earlier studies implicated the Ras components

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regulation provides a simple way for an animal to coordinate the ageing of different tissues. In addition, it may provide a way for life span to be changed rapidly during evolution. All metazoans, with their diverse life spans, evolved from a common precursor that probably did not live very long. Thus mutations that extend life span must have been important in evolution. The evolution of body pattern seems to be driven largely by changes in regulatory genes; the same could well be true of ageing. Having a system that regulates ageing allows changes in one or a few regulatory genes to create large differences in life span. During evolution, variants with unusual life spans might be able to enter new ecological niches that favour a different rate of ageing.

In addition to regulating life span, the *C. elegans* insulin/IGF-1 signalling pathway also regulates entry into an alternative developmental state called the dauer³⁹. The dauer is a growth-arrested, stress-resistant alternative larval stage that is induced by food limitation and crowding. Thus dauer formation is analogous to spore formation, or its milder vertebrate equivalent, hibernation. Because it allows animals to postpone reproduction until conditions improve, dauer formation has the same consequence for *C. elegans* that the response to caloric restriction has for vertebrates. The dauer state can be considered to be a puberty checkpoint, as it arrests growth just prior to reproductive maturation. Only young larvae can become dauers; once the animals have entered 'puberty', they no longer have this option.

The mutations in the insulin/IGF-1 pathway that affect adult life span are weak mutations^{51,60}. Strong mutations in the same genes cause young larvae to arrest as dauers. This indicates that low levels of endocrine signalling are sufficient to bypass the dauer checkpoint, whereas higher levels are required for normal life span. Dauers are thought to derive energy from stored fat, which they accumulate before entry into dauer. In order for animals to remain in the dauer state, *daf-2* levels must remain low³⁹. Thus this metabolic shift is analogous to the metabolic shift that occurs when insulin levels fall in humans, which also triggers the breakdown and metabolism of stored fat reserves.

Why do the insulin/IGF-1-pathway mutants live so long? In addition to regulating life span, the DAF-2 receptor also regulates adult fertility and movement⁵¹. Some *daf-2* mutants (called Class 2 alleles⁵¹) adopt a dauer-like, quiescent posture, have low fertility and lay eggs very late in life, even near death^{51,60}. These fertility and movement defects can be excluded as a cause of longevity, because another class of long-lived *daf-2* mutants (class 1 alleles) move normally and have normal brood sizes and reproductive schedules⁵¹. Another possibility is that a decrease in the overall rate of metabolism lengthens life span. This is an important issue, and the findings so far are controversial. In one study, the metabolic potential of insulin/IGF-1-pathway mutants was shown to be greater than that of wild-type animals⁶¹, arguing against this model. Likewise, in a second study⁶² the metabolic rate of a long-lived *age-1* (PI(3)K) mutant was not significantly different from wild type; however, a *daf-2* allele that had the same life span as the *age-1* allele had a very low rate of metabolism. Why was this? The *daf-2* mutant examined is known to behave as a healthy class 1 allele at 20 °C but as a quiescent class 2 allele at higher temperature^{60,51,63}. In this study⁶², this mutant grew to adulthood much more slowly than others have observed at 20 °C (refs 39, 40), suggesting that it was behaving as a class 2 allele, and thus expressing physiological defects unrelated to life-span extension. It will be interesting to determine the metabolic rates of true class 1 *daf-2* alleles.

daf-2 adults have dark intestinal pigmentation⁶⁴, which is correlated with a metabolic shift to fat production³⁹. However, intestinal pigmentation can be uncoupled from life span in genetic mosaic animals. *daf-2* functions non cell-autonomously to regulate both life span and intestinal metabolism⁶⁵. Some animals with *daf-2*⁻ intestines had normal intestinal pigmentation. Conversely, some animals in which part of the ectoderm was *daf-2*⁻ (but the intestine was *daf-2*⁺) had dark intestines. Many of these mosaics were long

lived. Significantly, in some classes of ectodermal mosaics, many or even all of the long-lived animals had normal intestinal pigmentation. Thus this metabolic shift is not required for life-span extension. Mutants in the insulin/IGF-1 pathway are known to be resistant to many forms of stress, including heat shock, ultraviolet (UV) light, hydrogen peroxide (H₂O₂) and paraquat⁶⁶⁻⁶⁸. It is possible that their increased ability to withstand oxidative stress is responsible for their longevity. The role of oxidative stress in ageing is discussed in more detail below.

A downstream signal

The insulin/IGF-1 pathway functions to regulate a downstream signal or hormone, because, in genetic mosaic analysis, removing *daf-2* activity from individual lineages can cause the whole animal to become a long-lived adult or to enter the dauer state⁶⁵. *daf-2* acts in lineages that produce ectodermal cells (neurons, skin), as well as lineages that produce predominantly endodermal and mesodermal cells to control both life span and dauer formation. *daf-2* also acts non cell-autonomously in the ectoderm (and also in internal tissues) to control intestinal metabolism and reproduction⁶⁵. For life span as well as dauer formation, ectodermal lineages appear to produce higher levels of downstream signal than do endodermal and mesodermal lineages. Surprisingly, loss of *daf-2* activity in tiny lineages that consist only of neurons can, at a low frequency, cause the entire animal to become a dauer. Likewise, a mosaic lacking *daf-2* only in three neurons (the ABalppap lineage) lived much longer than wild type⁶⁷. This indicates that *daf-2* functions in the nervous system to regulate dauer formation and life span. The fact that the insulin/IGF-1 pathway acts non cell-autonomously suggests that the targets of the DAF-16 transcription factor will be genes that encode, or regulate, a secreted signal (Fig. 3a,b).

Regulation of life span by sensory neurons

C. elegans can smell and taste many soluble and volatile compounds. Surprisingly, perturbations that decrease sensory perception, including mutations affecting components of sensory cilia or sensory signal-transduction pathways, as well as ablation of olfactory support cells, can extend mean life span up to 50% (ref. 69). These long-lived animals feed and reproduce normally (some actually have more progeny than normal), and they have normal rates of development. Genetic epistasis experiments indicate that sensory neurons influence life span, at least in part, by regulating the insulin/IGF-1 signalling pathway. A simple model is that an environmental signal, possibly a component of food or a pheromone, triggers the sensory neurons to secrete an insulin/IGF-1-like hormone that binds to the DAF-2 receptor and accelerates the ageing process (Fig. 3b). When sensory perception is prevented (or, in nature, when the environmental signal is absent), the hormone is not secreted, and the level of DAF-2 activity is decreased. This extends youthfulness and life span. This model is consistent with the finding that genes predicted to mediate secretion of insulin-like hormones act within the nervous system to regulate life span⁷⁰.

Regulation of life span by reproductive signals

The reproductive system, too, regulates ageing in *C. elegans*⁷¹. Killing the germ-line precursors causes the animals to live about 60% longer than normal. One might imagine that germ-line ablation extends life span simply by preventing reproduction. However, this seems unlikely because removing the entire reproductive system (the germ cells as well as surrounding somatic gonad) has no effect on life span⁷². A nuclear hormone receptor, DAF-12 (ref. 72), is required for the longevity of germ line-ablated animals, indicating that a steroid hormone may control the change in life span. This germ-line signalling system requires *daf-16*, but it seems to act independently of the DAF-2 receptor: if the germ cells are killed in *daf-2*⁻ mutants, then the animals remain active and healthy for a long time, and live four times as long as normal. Genetic studies indicate that the

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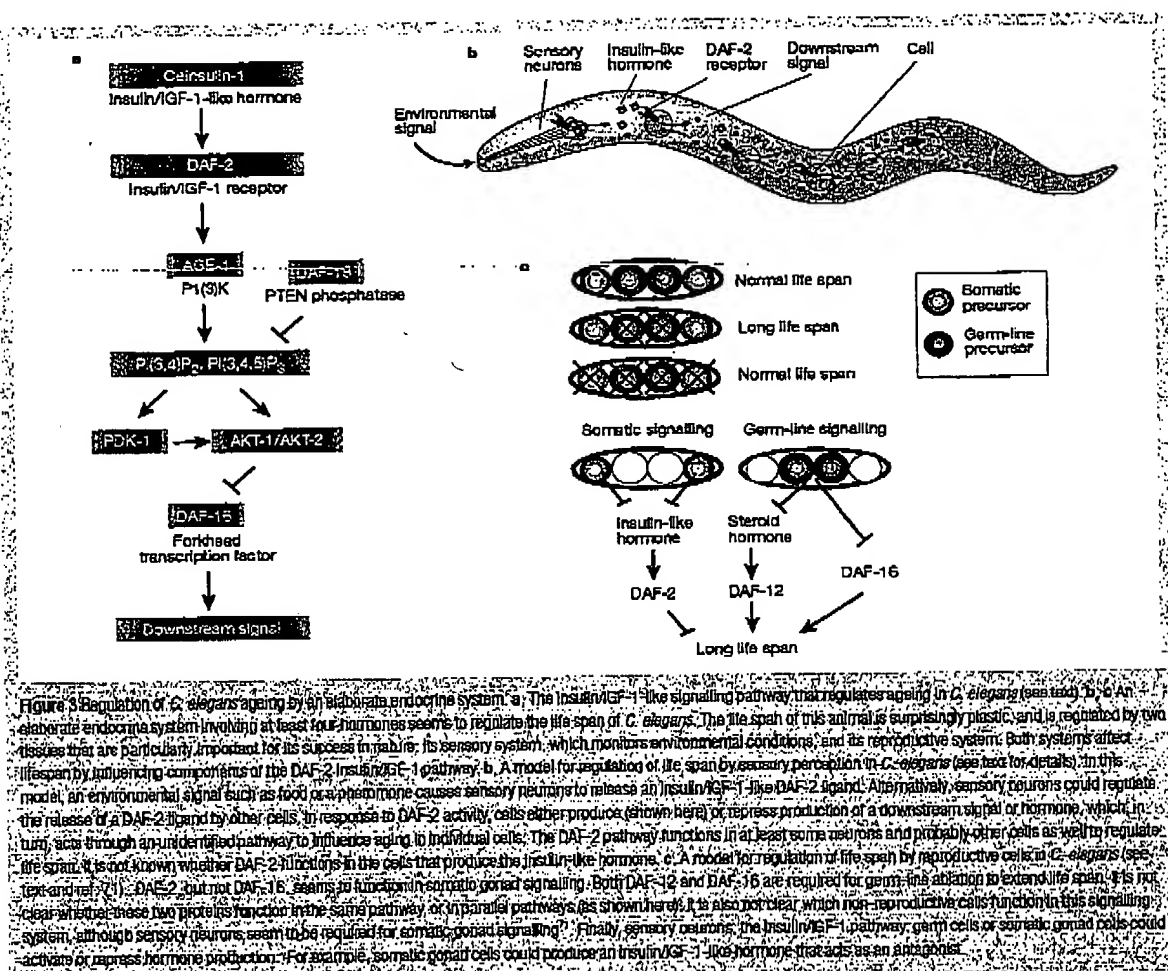


Figure 3 Regulation of *C. elegans* ageing by an elaborate endocrine system. **a**, The insulin/IGF-1-like signalling pathway that regulates ageing in *C. elegans* (see text). **b**, An elaborate endocrine system involving at least four hormones seems to regulate the life span of *C. elegans*. The life span of this animal is surprisingly plastic, and is regulated by two tissues that are particularly important for its success in nature: its sensory system, which monitors environmental conditions, and its reproductive system. Both systems affect life span by influencing components of the DAF-2/Insulin/IGF-1 pathway. **c**, A model for regulation of life span by sensory perception in *C. elegans* (see text for details). In this model, an environmental signal such as food or a pheromone causes sensory neurons to release an insulin/IGF-1-like DAF-2 ligand. Alternatively, sensory neurons could regulate the release of a DAF-2 ligand by other cells. In response to DAF-2 activity, cells either produce (shown here) or repress production of a downstream signal or hormone, which in turn, acts through an unidentified pathway to influence ageing in individual cells. The DAF-2 pathway functions in at least some neurons and probably other cells as well to regulate life span. It is not known whether DAF-2 functions in the cells that produce the insulin-like hormone. **c**, A model for regulation of life span by reproductive cells in *C. elegans* (see text and ref. 71). DAF-2, but not DAF-16, seems to function in somatic gonad signalling. Both DAF-12 and DAF-16 are required for germ-line ablation to extend life span. It is not clear whether these two proteins function in the same pathway or in parallel pathways (as shown here). It is also not clear which non-reproductive cells function in this signalling system, although sensory neurons seem to be required for somatic gonad signalling. Finally, sensory neurons, the insulin/IGF-1 pathway, germ cells or somatic gonad cells could activate or repress hormone production. For example, somatic gonad cells could produce an insulin/IGF-1-like hormone that acts as an antagonist.

somatic gonad produces a different signal that counterbalances the germ-line signal and extends life span, and suggest that this signal is, or regulates, a second insulin/IGF-1-like DAF-2 ligand (Fig. 3c). Surprisingly, somatic gonad signalling seems to be *daf-16* independent. Thus in the reproductive signalling system, *daf-2* can function independently of *daf-16* and vice versa.

Reproductive signalling could potentially coordinate an animal's schedule of reproduction with its rate of ageing. For example, if something were to delay the maturation of the germ line, then the animal might age more slowly. Because of this, it might remain youthful enough to bear progeny when the germ line reached maturity. Such a system could have been important in evolution, as animals with different life spans often have different reproductive schedules. Recent experiments in *Drosophila* complement these findings⁷³. Selective breeding experiments have given rise to strains of flies that produce progeny relatively late in life and are long lived, and strains that produce progeny at an earlier stage and are short lived. When the germ cells are killed, the life span of the short-lived strain is extended, and the difference between the life spans of the two strains is abolished. Thus the germ line can regulate ageing in *Drosophila*. The correlation between the time of reproduction and ageing in these two strains is intriguing. Perhaps the long-lived flies are long lived because germ-line development is delayed, causing them to produce a lower level of the germ-line 'ageing' signal.

Oxidative damage and ageing

Many findings in *C. elegans*, *Drosophila* and mice are consistent with the hypothesis that oxidative damage accelerates ageing, and that increased resistance to oxidative damage can extend life span (see review in this issue by Finkel and Holbrook, pages 239–247). So far, all of the long-lived *C. elegans* mutants tested have been found to be resistant to oxidative stress, as has a long-lived *Drosophila* mutant⁷⁴ (described below) and long-lived lines of flies produced by selective breeding^{75,76}. Mice and rats whose life spans have been extended by caloric restriction are also stress resistant⁷⁷, as are long-lived mouse mutants⁷⁸ (see below).

Conversely, mutations that increase oxidative damage can shorten life span. In *C. elegans*, mutations in *ctl-1*, a cytosolic catalase, shorten life span and prevent the life-span extension of *daf-2* mutants⁷⁹. *ctl-1* mutants seem to age more rapidly than normal, because they accumulate age-associated lipofuscin granules precociously. *ctl-1* expression also increases in *daf-2* mutants⁷⁹, as does expression of *sod-3*, which encodes a Mn-superoxide dismutase (SOD)^{80,81}. In addition, a mutation in *mev-1*, which encodes a subunit of the mitochondrial enzyme succinate dehydrogenase, accelerates the ageing process (by lipofuscin granule accumulation) and shortens life span⁸². This mutant exhibits increased sensitivity to oxygen. Recently, a drug known to act as an antioxidant was shown to extend the life span of *C. elegans* by up to 50% (ref. 82). In *Drosophila*,

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overexpression of Cu/Zn-SOD, either ubiquitously or specifically in motor neurons, extends life span by 40–50% without affecting metabolic rate or fertility^{33,34}.

Although oxidative damage is important in ageing, it seems unlikely to be the only cause. For one thing, the magnitude of life-span extension seen in certain *C. elegans* mutants, up to fourfold greater than in wild-type animals^{10,21,25}, is much greater than that produced by treatments that counteract oxidative damage. In addition, it is possible to uncouple life-span extension from stress resistance in calorically restricted yeast²¹. Other forms of damage, as well as cellular mechanisms that repair this damage, probably influence ageing as well.

Regulation of life span by a metabolic sensor

Another intriguing system that influences the life span of *C. elegans* is the *clk* gene system (reviewed in ref. 85). Mutations in these genes slow the rates of many processes, such as cell division, the rate of 'pumping' (feeding) and defecation. In addition, they lengthen adult life span by 15–30%. Genetic experiments indicate that the *daf* and *clk* mutants probably act in different pathways, because *daf-2 clk-1* double mutants live much longer than either single mutant. In addition, the longevity of *clk-1* mutants seems to be partly independent of *daf-16*. *clk-1* is homologous to the yeast *coq7* gene, which encodes a mitochondrial protein and is involved in the synthesis of ubiquinone. The *C. elegans* gene complements the yeast mutation, indicating that it has a similar biochemical activity. Although this suggests that *clk* mutants slow the 'rate of living' because they have a low metabolic rate, *clk-1* null mutants have nearly normal levels of mitochondrial respiration and do not seem to have reduced levels of ubiquinone.

Why are these mutants long lived? Because they live slowly, they may be long lived because their rate of metabolism is reduced, which, in turn, would be expected to reduce the rate of oxidative damage. In addition, because they pump slowly, they may be calorically restricted. Because *clk-1* mutants have normal rates of mitochondrial respiration, Hekimi and co-workers have argued that *clk-1* has a regulatory role in determining the 'rate of living'⁸³. Specifically, they propose that *clk-1*'s role is to report to the nucleus on the metabolic state of the mitochondria, causing nuclear genes to set the correct rates of behaviour, development and ageing. In the mutant, the nucleus does not receive this information from the mitochondria, and thus it sets the 'rate of living' too slowly. This is an interesting hypothesis, because it argues that the rate of mitochondrial respiration does not impact the 'rate of living' directly, but instead serves as a regulatory switch. This model is consistent with the observation that a small fraction of *clk-1* mutants undergo embryogenesis more rapidly than normal, suggesting deregulation rather than simple inhibition of metabolism.

Another interesting feature of this model is that it helps to explain the peculiar response of *clk-1* mutants to changes in temperature. Wild-type worms live, and age, more slowly at lower temperature than at high temperature. When two-cell embryos are transferred from high or low temperature to a moderate temperature, they promptly adjust their rate of development to the new temperature. *clk-1* mutants are unable to do this; instead, they undergo embryogenesis according to their temperature of origin. This indicates that the change in the rate of development that occurs with temperature is not simply a passive consequence of a change in thermal motion and biochemical reaction rates. Instead, this ability to conform to temperature must be under active regulation.

Life-span pathways in *C. elegans*

In *C. elegans*, at least four processes can influence life span: caloric restriction⁸⁶, signalling through the insulin/IGF-1 pathway, germline activity and the *clk* pathway. Although at this point it seems likely that the *clk*, insulin/IGF-1 and germline pathways are distinct, it is not clear whether any may function in the animal's response to caloric restriction. Certain *C. elegans eat* mutants (which do not eat

properly⁸⁷) are long lived⁸⁸. Genetic epistasis experiments indicate that these mutations and the *clk* mutations affect the same pathway⁸⁸. In contrast, *eat* mutations and *daf-2* mutations seem to affect different pathways⁸⁸. These findings suggest that the *clk* pathway, but not the insulin/IGF-1 pathway, participates in the response to caloric restriction. However, it is not clear that these *eat* mutants are actually calorically restricted, because they do not potentiate dauer formation in the way that actual food limitation does⁸⁷, and not all *eat* mutants are long lived. Caloric restriction in vertebrates causes insulin levels to fall. Thus it will be interesting to learn how mutants in the insulin/IGF-1 pathway respond to actual food limitation. Finally, overexpression of *tkr-1*, which encodes a tyrosine kinase, can extend life span in *C. elegans* by about 60%, without affecting development or fertility. These animals are also resistant to heat and UV light⁸⁹. This implies the existence of another signal-transduction pathway in the regulation of *C. elegans* life span.

A long-lived *Drosophila* mutant

Single-gene mutations can extend the life span of *Drosophila* as well as *C. elegans*. A partial loss-of-function mutation in the gene *methuselah* extends the average life span by about 35% (ref. 74). Thus in the wild type, this gene functions to shorten life span. Null alleles of *methuselah* die before reaching adulthood, indicating that the gene has an essential function during development. As mentioned earlier, *methuselah* mutants are resistant to a number of different stresses, including starvation, high temperature and paraquat, a free-radical generator. The *methuselah* gene encodes a putative G-protein-coupled seven-transmembrane-domain receptor. This suggests that a signal-transduction pathway regulates life span and stress resistance in this animal. G-protein-coupled receptors function in many types of signalling pathways. Because the *methuselah* protein has no close homologues with known function, it is difficult to predict in which type of pathway (for example, hormonal, sensory or glucose regulatory) it is likely to act.

Mutations that extend the life span of mice

A mutation in the gene encoding the protein p66^{lac} extends the life spans of mice by about 30% (ref. 78). p66^{lac} is an adaptor protein involved in the oxidative stress response. Normal cells in culture undergo apoptosis following treatment with agents that induce oxidative damage, such as H₂O₂, paraquat and UV light. In contrast, cells derived from mutant mice lacking p66^{lac} exhibit enhanced resistance to apoptosis following oxidative stress. The p66^{lac} protein is serine-phosphorylated following treatments that induce oxidative damage. This phosphorylation seems to be required for stress-induced apoptosis, because cells carrying p66^{lac} phosphorylation-site mutants are resistant to oxidative damage. The resistance to oxidative stress of p66^{lac} mutants can also be observed in whole-animal studies, as mice that lack p66^{lac} exhibit increased resistance to paraquat injection. It is not clear why p66^{lac} mutants are long lived. One possibility is that in the wild type, the loss of cells resulting from stress-induced apoptosis accelerates ageing. Because this apoptosis is blocked in p66^{lac} mutants, life span is extended. However, it is also possible that loss of p66^{lac} reduces the amount of damage induced by oxidative damaging agents in the first place. This would have the effect of increasing the health of individual cells, and it would also reduce apoptosis. In both models, additional cells would survive in the mutant mice, but in the latter case cellular components would undergo less oxidative damage. Finally, it is important to note that resistance to oxidative stress in these animals need not be the cause of their longevity. The correlation is intriguing, but not a demonstration of causality.

As in *C. elegans*, endocrine signalling seems to regulate ageing in mice. Mutations in genes that inhibit the development of the pituitary gland cause dwarfism and life-span extension. One such gene, defective in the Ames dwarf mice, encodes Prophet of Pit-1 (PROF1), a homeodomain protein that is expressed specifically in

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the embryonic pituitary gland and is required for expression of Pit-1, which, in turn, is required for pituitary development³⁰. Ames dwarf mice lack several hormones, including growth hormone, prolactin and thyroid-stimulating hormone, and they live approximately 50% longer than normal³¹. Why the Ames dwarf mice are long lived is not clear. For example, it is not known whether their small body size is required for their longevity, and which hormone (or hormones) defective in the mutant may regulate life span. Interestingly, animals that overexpress growth hormone exhibit signs of accelerated ageing^{32,33}, and growth hormone-receptor mutants seem to have extended life spans³². Like calorically restricted mice, the dwarf mice have a low body temperature³⁴. In addition, they have elevated levels of catalase and Cu/Zn SOD, and decreased levels of reactive oxygen species^{35,36}. These features are consistent with their longevity being due to reduced metabolic rate or oxidative damage. However, a report investigating the level of these defence proteins in the hypothalamus³⁶ complicates this interpretation. In this tissue, both the long-lived dwarf mice as well as the short-lived mice overexpressing growth hormone have increased levels of catalase and Cu/Zn SOD.

Conservation

Many biological processes are conserved widely throughout the animal kingdom. Is this true of ageing? Caloric restriction can extend life span in a wide range of organisms; thus this seems like a good candidate for an evolutionarily conserved ageing pathway. Little is known about the genes that regulate life-span extension in response to caloric intake. In this regard, it will be particularly interesting to learn whether the yeast SIR2 histone deacetylase pathway (described above) regulates ageing in metazoans and, if so, whether it acts in the response to caloric restriction. In addition to caloric restriction, there are indications that both germ-line activity and oxidative damage influence ageing in both flies and worms, and that oxidative damage might influence ageing in vertebrates as well (see review in this issue by Finkel and Holbrook, pages 239–247). In addition, signalling from the mitochondria may regulate life span in at least two organisms, *C. elegans* and yeast. Does insulin/IGF-1 signalling regulate life span in animals other than *C. elegans*? Mutations affecting insulin/IGF-1 signalling have been discovered in flies, but their effect on life span is not known. In humans, insulin-receptor mutations cause diabetes rather than longevity; however, these mutations lead ultimately to a severe loss of insulin production and so may not mimic the weak signalling mutations of *C. elegans*. Further complicating the situation, vertebrates have at least three different insulin-receptor family members, and it is becoming clear that different branches of the insulin/IGF-1 signalling network control different aspects of physiology and metabolism. Possibly one branch of this network controls ageing. This seems particularly plausible given the effects of endocrine perturbation on ageing in dwarf mice, especially since the dwarf mice have reduced levels of insulin³⁵. If ageing does prove to be regulated hormonally in humans, then it may be possible to extend youthfulness and increase the quality of old age with drugs or hormones that perturb the endocrine system.

The long-lived *Drosophila methuselah* mutant, which is defective in a putative G-protein-coupled receptor, indicates that cell-cell communication or endocrine signalling may influence the life span of flies, but it is not clear whether *methuselah* homologues regulate life span in other animals. A similar situation exists with the *C. elegans tkr-1* gene, which encodes a tyrosine kinase receptor that appears to extend life span³⁷. Ultimately it seems likely that each known life-span gene will be found to act in one of several pathways that regulate ageing, and that at least some of these pathways will be highly conserved. These are still early days; the screens for life-span mutants are not saturated in any organism.

Diseases of ageing

Ageing in humans is manifest not only by the stereotypical changes in phenotype but also by a large increase in the onset of many diseases.

Can these diseases be thought of as an integral part of the ageing process; that is, would some anti-ageing intervention slow their onset? A useful example to consider may be cancer. Clearly, like ageing, the progression of many cancers is a time-dependent process. The role of somatic mutations in the pathways of cancer development has been amply documented (see review in this issue by DePinho, pages 248–254). Therefore, the development of at least certain cancers would seem to be mechanistically separate from ageing. However, a link between ageing and cancer is suggested by the delay of cancers in calorically restricted mice, and by the striking correlation between physiological age and the likelihood of cancer in animals with very different life spans. This suggests that any treatment that slows the ageing process, whether it acts directly on hormone signalling, gene silencing or oxidative stress, may have the potential to delay cancer and possibly other diseases of ageing.

Summary

The field of ageing research has been completely transformed in the past decade. It is now widely accepted that the ageing process, like most biological processes, is subject to regulation and can be studied using classical genetics. When single genes are changed, animals that should be old stay young. In humans, these mutants would be analogous to a ninety year old who looks and feels forty-five. On this basis we begin to think of ageing as a disease that can be cured, or at least postponed. This paradigm shift is due largely to the analysis of single-gene mutations that influence ageing in model organisms. The field of ageing is beginning to explode, because so many are so excited about the prospect of searching for — and finding — the causes of ageing, and maybe even the fountain of youth itself.

Note added in proof. Recently, Wolkow *et al.*³⁷ have shown that expression of the *C. elegans daf-2* receptor or *age-1* PI(3)K only in neurons can confer normal life span, and that expression of *daf-2* only in endoderm has a significant, but lesser, effect. These findings are in accord with previous mosaic analysis⁴⁸. An important caveat is that the levels of DAF-2 and AGE-1 produced in these transgenic animals may differ from endogenous levels, possibly altering the level of downstream signal. Wolkow *et al.* also demonstrate that expressing *daf-2* or *age-1* only in neurons can be sufficient for normal fat metabolism in the intestine. This is consistent with earlier findings that *daf-2* activity in the ectoderm can be necessary and sufficient for normal intestinal pigmentation⁴⁸, although it should be noted that genetic mosaic animals with a nervous system that is almost completely wild type but internal tissues that are *daf-2*⁺ often have a *Daf-2*⁺ intestinal phenotype. Finally, the new study reported that the altered intestinal metabolism of insulin/IGF-1 pathway mutants is not required for longevity, as had been shown previously by analysing intestinal pigmentation and life span in genetic mosaics⁴⁵ (see text). □

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